Formation of Folate Enzymes During the Growth Cycle of Bacteria

II. Activity of Serine Hydroxymethyltransferase during the Lag, Acceleration and Exponential Phases of Growth of *Escherichia coli*, *Streptococcus faecalis*, *Lactobacillus arabinosus* and *Streptococcus thermophilus*

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The formation of serine hydroxymethyltransferase in the active growth phases of *Escherichia coli*, *Streptococcus faecalis*, *Lactobacillus arabinosus* and *Streptococcus thermophilus* has been studied. The results suggest that these bacteria differ notably from each other as regards the occurrence of maxima in enzyme activity during the growth cycle. Investigations concerning especially the lag phase of growth showed a clear increase in the activity of serine hydroxymethyltransferase during that growth period. On the basis of this finding serine hydroxymethyltransferase should be considered a typical "lag phase enzyme".

The enzyme serine hydroxymethyltransferase (systematic name according to the Report of the Commission on Enzymes:[1] L-serine tetrahydrofolate 10-hydroxymethyltransferase, number 2.1.2.1.), also known as serine aldolase, serine transhydroxymethylase, and serine hydroxymethylase, catalyzes the interconversion of serine and glycine, a reaction in which also pyridoxal phosphate is involved. The reaction may be represented as follows:

L-serine + tetrahydrofolate (FH_4) $\rightleftharpoons$ glycine + 10-hydroxymethyltetrahydrofolate (10-hydroxymethyl-FH_4)

Both the reaction and the enzyme have been subjects of numerous investigations, and excellent reviews covering this field are available, *e.g.* Refs.[2–7]

In two previous papers from this laboratory [8,9] it was demonstrated that certain strains of lactic acid bacteria and *Escherichia coli* produce large amounts of folic acid during growth (microbiological assays, test organism *Streptococcus*

faecalis R). Especially the finding that considerable variations occur in the folic acid content of the cells during the first growth phase, the lag phase, led us to study folate enzymes and their activities during the active growth phases of bacteria. The formation of tetrahydrofolate dehydrogenase during the lag and acceleration phases of growth of *Streptococcus faecalis* R was reported recently from this laboratory.[10]

The work described in this paper shows that marked variations occur in the activity of serine hydroxymethyltransferase in cells of *Escherichia coli*, *Str.faecalis* R, *Lactobacillus arabinosus* 17—5 and *Streptococcus thermophilus* during the active growth phases of the organisms. Part of the findings have been briefly reported earlier.[11]

EXPERIMENTAL

Organisms and growth media. Escherichia coli, strain U5—41, was cultured with monthly transfers in an agar medium which contained 2.0 g of glucose, 1.4 g of KH_2PO_4, 100 mg of trisodium citrate, 200 mg of ammonium sulphate, 220 mg of Casamino acids (Difco), 20 mg of $MgSO_4 \cdot 7H_2O$, 10 mg of tryptophan, and 2.0 g of agar (Difco) in 100 ml of distilled water. The medium was adjusted to pH 6.7—6.8 and autoclaved 10 min at 105°. The inoculated agar slant was incubated 24 h at 37° and stored in a refrigerator at + 2°.

The inoculum medium for *E. coli* contained 1.0 % glucose, 1.0 % trisodium citrate, 0.5 % Bacto-tryptone (Difco), and 0.5 % yeast extract (Difco). The pH of the medium was adjusted to 6.7—6.8 and the medium was autoclaved 10 min at 115°.[12]

The growth medium for *E. coli* contained glucose, 1.0 g, KH_2PO_4, 700 mg, trisodium citrate, 50 mg, $(NH_4)_2SO_4$, 100 mg, NH_4Cl, 100 mg, $MgSO_4 \cdot 7H_2O$, 10 mg, and distilled water, 100 ml. L-Glutamic acid was omitted. The medium was adjusted to pH 6.7—6.8 with 1 N NaOH and boiled shortly before the experiment started.[13]

Str. faecalis R (ATCC 8043) and *Lb. arabinosus* 17—5 (ATCC 8014) were maintained as stick cultures in GSHT-agar containing 1.0 % glucose, 1.0 % trisodium citrate, 0.5 % Bacto-tryptone (Difco), 0.5 % yeast extract (Difco), and 2.0 % Bacto-agar (Difco). The pH of the basal medium was 6.7—6.8. Sterilization was performed at 110° for 10 min. The organisms were transferred to fresh agar media every two weeks.

The inoculum medium for *Str. faecalis* R and *Lb. arabinosus* 17—5 contained 2.0 g of lactose, 0.5 g of glucose, 0.5 g of sucrose, 0.5 g of yeast extract (Difco), 0.25 g of gelatin, and 70 mg of ascorbic acid in 100 ml of distilled water.

The growth medium for *Str. faecalis* R was prepared from Bacto Folic Acid Assay Medium (Difco), 37.5 g of which was dissolved in 1000 ml of distilled water. To the solution was added 5 μg of pteroyl-L-glutamic acid. The growth medium for *Lb. arabinosus* 17—5 contained 37.5 g of the Bacto Biotin Assay Medium and 0.25 μg of biotin diluted to 1000 ml with distilled water.

The *Streptococcus thermophilus* strain KQ was maintained and cultivated in medium ScQ the composition of which is shown in Table 1. Before inoculation the cells were harvested and washed with 0.9 % sodium chloride solution. The washed cells were suspended in 50 ml of saline, whereupon a slightly opaque suspension resulted, two drops of which was then used to inoculate 5 ml of growth medium.

Amino acid variants of the *Str. thermophilus* strain KQ were obtained by centrifuging the strain in 5 ml of medium ScQ containing 0.5 mmole per litre of the amino acid in question (L-form). The growth temperature was 42°, and the growth time varied between 18 and 48 h depending on how soon a clearly noticeable growth resulted. After the cells had been centrifuged and washed with sterile saline, they were transferred to fresh ScQ media with the same additions as before and the incubation was repeated. All in all, the organisms were transferred four times, and the fifth and final incubation lasted 18—20 h. The resulting variants were stored in their growth media at + 4° with biweekly transfers. Three different variants were prepared by this method, the glycine variant (Str KQGly), the serine variant (Str KQSer), and the threonine variant (Str KQThr).

Table 1. Growth medium (medium ScQ) for lactic acid bacteria.

Compound	Amount
Sucrose	5.0 g
Lactose	5.0 »
β-Glycerophosphate	5.4 »
Natrium acetate, trihydrate	200 mg
Ascorbic acid	200 »
Ammonium chloride	267 »
L-Glutamic acid	147 »
L-Cysteine HCl	88 »
Potassium chloride	748 »
Magnesium sulphate	304 »
Thiamine	1 »
Riboflavin	1 »
Nicotinic acid	1 »
p-Aminobenzoic acid	1 »
Pyridoxine HCl	1 »
Calcium pantothenate	0.5 »
Biotin	1 μg
H_2O (twice distilled) pH 6.8	ad 1000 ml

Procedure. Before an experiment *E. coli*, *Str. faecalis* R or *Lb. arabinosus* 17—5 was transferred from the agar or stick culture to 5 ml of inoculum medium, where each grew 5—6 h at 37°. The culture was poured aseptically into 1 litre of the same medium and incubated overnight at 37°. The cells were centrifuged and washed three times with a 0.9 % sodium chloride solution. After the washing, the cells were transferred to 1 litre of the growth medium prepared for the organism in question and warmed to 37°, at which temperature the culture was then incubated. During the growth of *E. coli* the medium was constantly mixed with an electric stirrer. Samples were withdrawn at intervals, and their optical densities were measured with a Klett-Summerson colorimeter employing filter 62. The samples withdrawn during the lag phase contained about 10 mg dry weight of cells, and those taken during the exponential phase about 5 mg. The cells were centrifuged (at 3500 rpm), dried with cold acetone, and autolyzed overnight in 1 ml of 0.001 M phosphate buffer at 2°.

The medium was not stirred during the growth of *Str. faecalis* R and *Lb. arabinosus* 17—5. Samples 10 ml in volume were withdrawn, centrifuged (Servall SS-1, at 6000 rpm), washed twice with 0.9 % sodium chloride solution and frozen to —20°. The activity was preserved unchanged overnight in the frozen cells. Before the activity determinations, the cells were suspended in 100 μl of 0.005 M phosphate buffer of pH 7.4 and frozen and thawed twice.

When *Str. thermophilus* strains were used as test organisms, 5 ml of culture medium was transferred to 200 ml of ScQ growth medium. After incubation at 42° for 16—17 h, the cells were harvested by centrifuging (Servall SS-3, 4000 × *g* for 10 min), washed and suspended in 400 ml of ScQ medium. The temperature of incubation was 42°. In order to prolong the lag phase of growth, the inoculum was cooled (to + 2°) before centrifuging in some experiments.

The first cell sample was withdrawn immediately after inoculation and the following samples during the different growth phases. The cells were cooled in ice-water, centrifuged (Servall SS-1, 5000 × *g* for 10 min), washed with saline and centrifuged again (Servall SS-1, 4000 × *g* for 15 min). The cells were suspended in 100 μl of 0.1 M potassium phosphate buffer (pH 7.4) and stored at —20° if not used immediately. Before the enzyme activity determinations the cells were frozen and thawed three times.

Enzyme assay. A 300-μl volume of the reaction mixture contained 0.1 M phosphate buffer (pH 7.4), DL-serine 20 μmoles, NAD 0.02 μmole, pyridoxal phosphate 0.02 μmole, FH_4 0.6 μmole, and, according to determinations made during the lag phase of growth, 0.5 mg dry wt. of cells or, according to determinations made during the exponential phase, 1.0 mg dry wt. Pipetting was performed at + 1°. In order to prevent FH_4 from oxidizing, the reaction mixture was covered with petroleum ether. The reaction time was 4 h. The reaction was stopped by adding 200 μl of 6 % $HClO_4$ to the solution. The precipitated protein was removed by centrifugation (2500 rpm).

A 100-μl volume of the clear supernatant was pipetted onto Whatman No. 1 paper (60 × 60 cm). The glycine formed in the reaction was separated by paper chromatography using the descending technique and pyridine-water 65:35 as solvent. The R_F values were 0.41 for glycine and 0.51 for serine.[14] After the paper was stained with ninhydrin, the glycine spots were cut out and extracted with 5 ml of methanol. The intensity of the colour was measured with a Klett-Summerson colorimeter employing filter No. 56. The glycine contents of the extracts were calculated on the basis of standard lines drawn for known amounts of glycine. Figs. 1 and 2 show reaction rate curves obtained as described above.

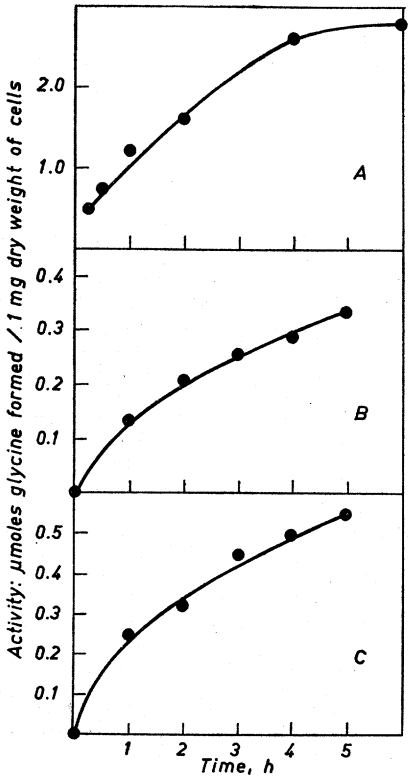

Fig. 1. Serine hydroxymethyltransferase reaction rate curves for different test organisms. A. *E. coli.* B. *Str. faecalis.* C. *Lb. arabinosus.*

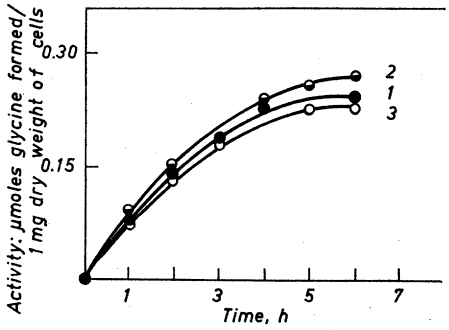

Fig. 2. Serine hydroxymethyltransferase reaction rate curves for different Str KQ variants. 1. Str KQ. 2. Str KQSer. 3. Str KQThr and Str KQGly.

The non-enzymatic fixation of serine to FH_4 was studied by passing the reaction mixture, which had been incubated without enzymes for 4 h at 37°, through a Dowex-1-chloride column, decomposing the serine present in the eluate with 0.3 M sodium meta-periodate, and determining the formaldehyde with the Nash reagent.[15]

FH_4 was prepared by the method of Kisliuk,[16] except that the filtration was performed in a nitrogen atmosphere and the filtrate was passed directly into peroxide-free ether containing 1 % of 2-mercaptoethanol at −30°. FH_4 was preserved under this ether. For the determination the FH_4 was dissolved under ether into a small volume of 0.1 M phosphate buffer of pH 7.4 from which the air had been removed in a vacuum and which contained 1 % of 2-mercaptoethanol. The FH_4 content of the solution was determined with a Beckman DU spectrophotometer at a wavelength of 298 mμ ($\varepsilon_{298} = 1.9 \times 10^6$ cm^2 $mole^{-1}$). The FH_4 prepared in this way had $\lambda_{max} = 298$ and contained 70 % of the theoretical amount of nitrogen as determined by the method of Kjeldahl.

RESULTS

When investigating the formation of the enzyme during the growth of the test organisms, we divided the growth cycle into phases according to Monod's [17] definitions. These phases are the lag, acceleration, exponential, retardation and stationary phases. In the present paper only the lag phases of *E. coli*, *Lb. arabinosus* and the *Str. thermophilus* strains are considered. The lag phase of *Str. faecalis* R was so short that it could not be observed in the experiments.

Fig. 3 shows the formation of the enzyme during the lag phase of *E. coli*. At the beginning of the phase, the activity was 0.4 μmole of gly/mg/4 h.

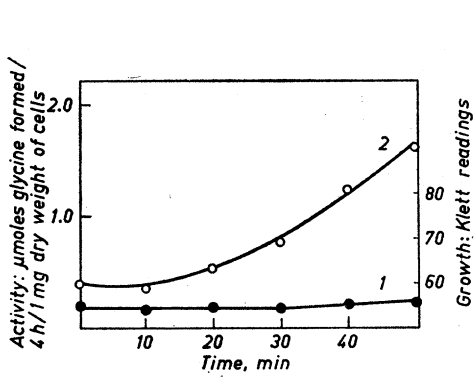

Fig. 3. Serine hydroxymethyltransferase activity during the lag phase of *E. coli*. 1. Growth curve. 2. Activity curve.

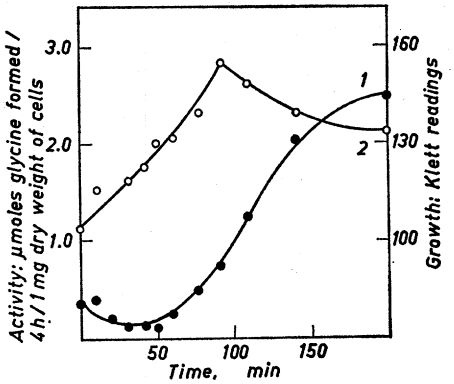

Fig. 4. Serine hydroxymethyltransferase activity during the growth of *E. coli*. 1. Growth curve. 2. Activity curve.

Towards the end of the phase, or 50 min after growth commenced, the activity was 1.6 μmoles of gly/mg/4 h. Accordingly, a 4-fold increase occurred during the lag phase of growth. Fig. 4 shows the enzyme formation mainly during the exponential and retardation phases. The activity continues to increase after the lag phase. At the beginning of the acceleration phase it was 1.75 μmoles of gly/mg/4 h in this experiment. A maximum activity of 2.8 μmoles of gly/mg/4 h was reached when 60 % of the exponential phase was over.

The increase in activity was thus 1.6-fold during the exponential phase. The decrease following the maximum was not due to any decrease in pH because a similar activity curve was obtained when the pH was kept constant during the growth by adding 5 M sodium hydroxide.

In connection with this work the possibility that certain compounds (glycine, DL-serine, DL-threonine, pteroyl-L-glutamic acid, sarcosine, adenine and xanthine) connected with the metabolism involving the serine-glycine conversion affect the activity of serine hydroxymethyltransferase in *E. coli* was also investigated. For this purpose 1—5 μmoles of the compounds studied were added to the growth medium of *E. coli*. None, however, of the investigated compounds had any decisive effect on the activity of the enzyme. Glycine and DL-threonine increased the activity during the lag phase. During the exponential phase pteroyl-L-glutamic acid and sarcosine increased the activity, while adenine and xanthine decreased it, but these changes were all so insignificant that no absolute values can be presented.

Under the experimental conditions serine and FH_4 did not react without enzymes present (Table 2).

Table 2. Effect of serine on FH_4 when the reaction mixture lacked serine hydroxymethyltransferase. The reaction mixture contained 30 μmoles of DL-serine, 0.02 μmole of NAD, 0.02 μmole of pyridoxal phosphate and 1.5 μmoles of FH_4 in a 0.05 M phosphate buffer of pH 7.4. The volume of the reaction mixture was 5.0 ml and the mixture was incubated at 37°. Samples 1 ml each were withdrawn after 0, 1, 2, 3, and 4 h. The samples were run through a Dowex-1-chloride column (1 × 10 cm, 100 mesh). The resin was washed with 10 ml of distilled water, which was then added to the solution that had passed through the column. A 0.8-ml volume of 0.3 M $NaIO_4$ was added to the eluate. After 30 min the formaldehyde formed from serine was determined with the Nash reagent.[15,16] The intensity of the colour was measured with a Klett-Summerson colorimeter employing filter No. 42. Two parallel determination were made on each sample.

Hours	Colorimeter readings	Control (Serine, 30 μmoles)
0	172	176
	175	175
1	174	
	175	
2	175	
	173	
3	175	
	177	
4	174	
	175	

In the study of the formation of serine hydroxymethyltransferase in *Str. faecalis* R, the organism was precultivated for such a short time (9 h) that no lag phase was observed during the growth.[10] When the precultivation was prolonged until a lag phase occurred, the results of the experiments became uncertain, probably because of the resulting large amount of dead cells.

When growth began in a fresh medium the serine hydroxymethyltransferase activity of the cells decreased 54—55 % during the first hour. Maximum activity was reached when 33 % of the active growth phases of the cells was over. After the maximum the activity decreased and then followed another slight increase which came to an end when the stationary phase began (Fig. 5).

If the pH was kept constant during the growth by base additions, the growth was clearly accelerated (Fig. 6). When the stationary phase began,

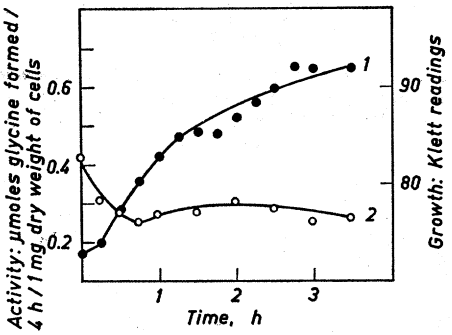

Fig. 5. Serine hydroxymethyltransferase activity during the growth of *Str. faecalis* R. 1. Growth curve. 2. Activity curve.

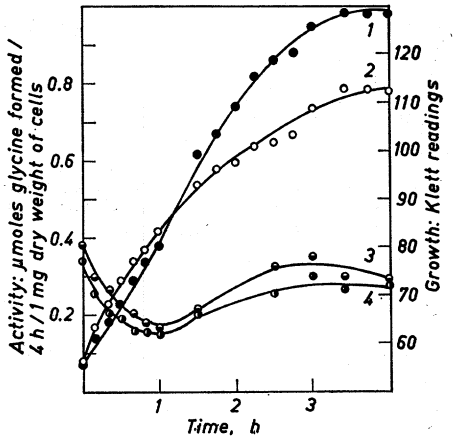

Fig. 6. Serine hydroxymethyltransferase activity during the growth of *Str. faecalis* R in a neutralized and in an unneutralized medium. Growth curves: 1. Growth in a neutralized medium (pH 6.8). 2. Growth in an unneutralized medium (pH 4.5 at the end of growth). Activity curves: 3. Activity in a neutralized medium (pH 6.8). 4. Activity in cells grown in an unneutralized medium (pH 4.5 at the end of growth).

the dry weight of cells grown in the neutralized medium was 13 % higher than that of cells grown in an unneutralized medium.

With *Lb. arabinosus* 17—5 the activity increased some 25 % during the lag phase of growth (Fig. 7). It remained constant during the acceleration phase and again increased during the exponential phase, this time 40 % (Fig. 8). The activity was a maximum when 60 % of the exponential phase was over. At the beginning of the stationary phase the enzyme activity was again the same as it was at the beginning of the lag phase or 0.5 µmole of gly/mg/4 h. The maximal activity in the exponential phase was 0.92 µmole of gly/mg/4 h, which means a 1.84-fold total increase.

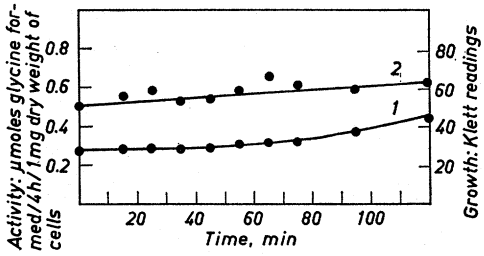

Fig. 7. Serine hydroxymethyltransferase activity during the lag phase of *Lb. arabinosus* 17—5. 1. Growth curve. 2. Activity curve.

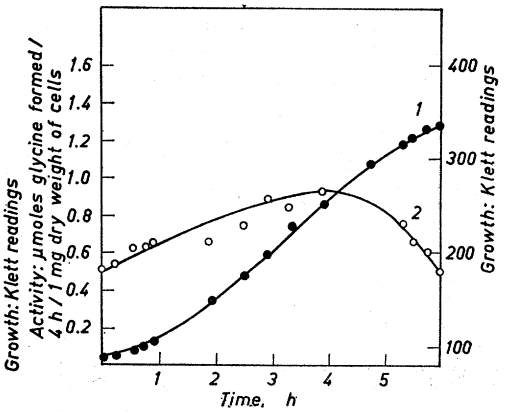

Fig. 8. Serine hydroxymethyltransferase activity during the growth of *Lb. arabinosus* 17—5. 1. Growth curve. 2. Activity curve.

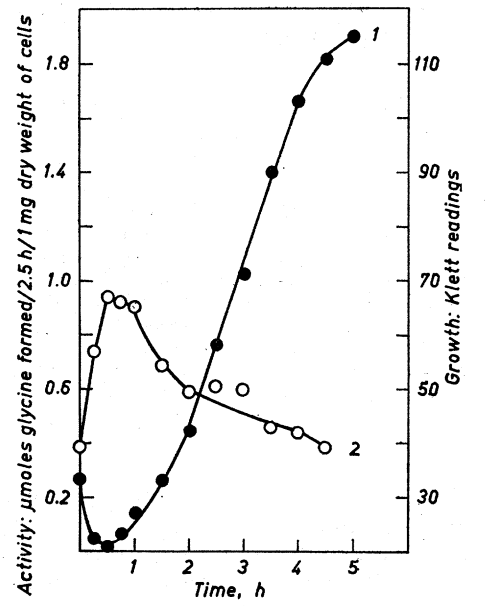

Fig. 9. Serine hydroxymethyltransferase activity during the growth of Str KQ. 1. Growth curve. 2. Activity curve.

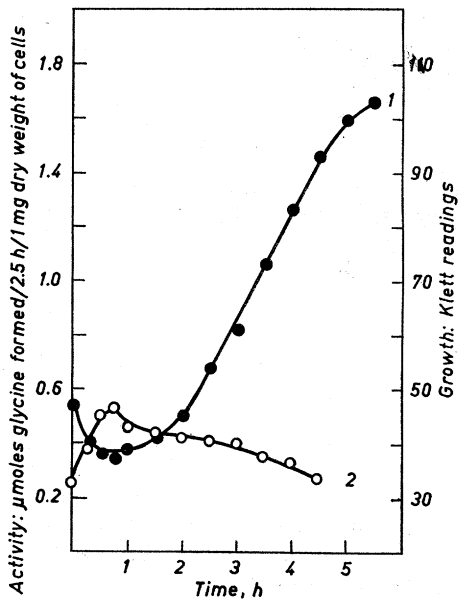

Fig. 10. Serine hydroxymethyltransferase activity during the growth of Str KQGly. 1. Growth curve. 2. Activity curve.

The variations in serine hydroxymethyltransferase activity during the growth cycles of *Str. thermophilus* KQ (Str KQ) and its glycine, serine and threonine variants (Str KQGly, Str KQSer, and Str KQThr, respectively) were also investigated. As can be seen from Figs. 9, 10, 11, and 12, the serine hy-

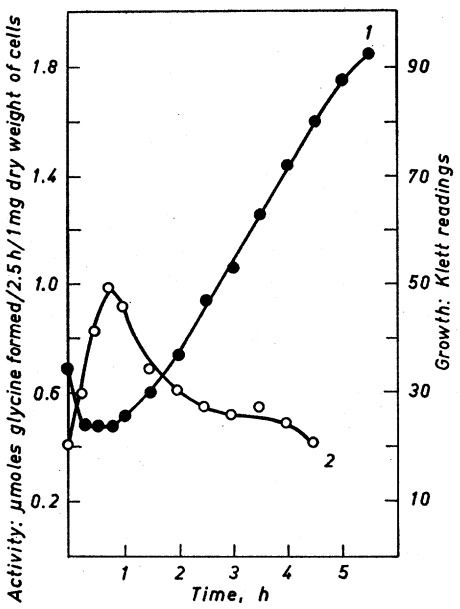

Fig. 11. Serine hydroxymethyltransferase activity during the growth of Str KQSer. 1. Growth curve. 2. Activity curve.

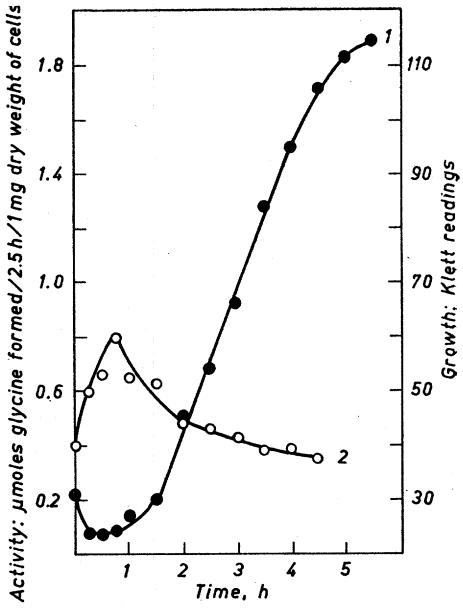

Fig. 12. Serine hydroxymethyltransferase activity during the growth of Str KQThr. 1. Growth curve. 2. Activity curve.

droxymethyltransferase activity began to increase in all tested strains at the very beginning of the lag phase and continued to do so up to the end of the phase or to the beginning of the acceleration phase. It is interesting to note that the enzyme activity rose to a maximum before the logarithmic phase and that the activity decreased during the logarithmic phase until at the end of the phase it was approximately equal to the activity at the beginning of the lag phase. When the enzyme maxima for the different strains are considered, it appears that there are notable differences between the three organisms: Str KQSer had the highest maximum activity level, and Str KQGly the lowest. In additional experiments it was found that the variation of pH during the growth did not affect the shape of the enzyme activity curves.

DISCUSSION

Several different procedures for determining serine and glycine have been presented in connection with investigations of the serine hydroxymethyltransferase reaction. The addition of formaldehyde to FH_4 has been shown to occur by using labeled formaldehyde.[16] Formaldehyde and serine can also be determined colorimetrically[15]. Spizizen *et al.*[18] have studied the reaction by measuring the amount of produced 5,10-methylene-FH_4 spectrophotometrically after an acid had changed it into 5,10-methenyl-FH_4. Blakley [19] determined serine manometrically. In their studies of the cofactor requirements

of the reaction, Wright *et al.*[20] separated the produced glycine by paper chromatography using a mixture of acetone-triethylamine-water, 80:5:15, as solvent. Since we used a crude cell extract we considered the paper chromatographic separation the best procedure to determine glycine. We also noticed that the solvent pyridine-water, 65:35, separated serine from glycine better than did acetone-triethylamine-water. The standard error of the procedure was 8.3 %.

In this study Monod's definitions [17] of the bacterial growth phases have been followed. In accordance with these definitions we have taken the lag phase to extend over the period before the beginning of growth when no cell division takes place, and the acceleration phase over the period during which cell division occurs with increasing speed, while during the logarithmic phase the rate of cell division is constant.

When comparing the variations in serine hydroxymethyltransferase activity during the growth cycles of the strains, one notices that the investigated lactic acid bacteria differ rather markedly from each other in this respect. With *Lb. arabinosus* 17—5 and with the *Str. thermophilus* strains the enzyme activity increased from the very beginning of the lag phase, but no such increase was observed with *Str. faecalis* R because the cell division started as soon as the strain had been transferred to a new growth medium. In any case, a decrease in the enzyme activity of the *Str. faecalis* R cells was observed from the beginning of growth and during the first active growth phases, especially in the acceleration and early logarithmic ones. Only in the later part of the logarithmic phase and in the retardation phase was the enzyme activity again observed to increase slightly.

Interesting differences were also observed in the serine hydroxymethyltransferase activity of *Str. thermophilus* strains and *Lb. arabinosus* 17—5. With the first strain the activity increase was very steep and a maximum was reached as early as in the lag phase or at the beginning of the acceleration phase, while the enzyme activity maximum for *Lb. arabinosus* 17—5 did not appear before the middle of the logarithmic phase.

At this stage of the work, however, it is difficult to draw any conclusions about the factors that cause these remarkable differences in the serine hydroxymethyltransferase activities of the investigated lactic acid bacteria. Our future investigations on the enzyme activity will center on compounds which may act as inhibitors or as repressors of the activity.

Wright [21] has shown with *Clostridium HF* that NAD inhibited the serine hydroxymethyltransferase reaction when FH_4 was used as folic acid coenzyme, but NAD was essential when the FH_4 was substituted by a triglutamate derivative of leucovorine CoC I which she had isolated from *Clostridium cylindrosporum*. However, our experiments show that NAD is essential also when the coenzyme is FH_4 if a crude preparation from *E. coli* is used as enzyme.

In this study the activity of the serine hydroxymethyltransferase of *E. coli* was found to be definitely stronger than that of the lactic acid bacteria. The activity values are higher throughout with *E. coli*, and the increase in activity is steeper than, for example, with *Lb. arabinosus* 17—5.

The activity of *E. coli* is probably somewhat higher than our values indicate. Under the experimental conditions, the serine used as substrate is deaminated

under the influence of the strong serine dehydratase (E.c. 4.2.1.13.) contained in the cells.[22] In our experiments a large amount of serine was used in the reaction mixture in order to eliminate as effectively as possible the lowering of the serine hydroxymethyltransferase activity that could have been caused by serine dehydratase. Apparently, also the fact that the pH optimum of serine dehydratase is 9.3 worked in the same direction, too.[23] However, the shape of the activity curve for serine hydroxymethyltransferase is not likely to have been much changed by serine dehydratase as only very slight changes occurred in the activity during the lag and exponential phases of *E. coli*, as has previously been found in this laboratory.[24,25]

Finally, the results presented indicate that serine hydroxymethyltransferase must be included among those enzymes the activity of which increases in the cells from the beginning of the lag phase, and which we have called previously "lag phase enzymes".[10]

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